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# Dysregulation of Innate Immunity in Hepatitis C Virus Genotype 1 *IL28B*-Unfavorable Genotype Patients: Impaired Viral Kinetics and Therapeutic Response

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**Recent studies have shown that a single-nucleotide polymorphism upstream of the interleukin-28B (*IL28B*) gene plays a major role in predicting therapeutic response in hepatitis C virus (HCV)-infected patients treated with pegylated interferon (PEG-IFN)/ribavirin. We sought to investigate the mechanism of the *IL28B* polymorphism, specifically as it relates to early HCV viral kinetics, IFN pharmacokinetics, IFN pharmacodynamics, and gene expression profiles. Two prospective cohorts (human immunodeficiency virus [HIV]/HCV-coinfected and HCV-monoinfected) completing treatment with IFN/ribavirin were enrolled. Patients were genotyped at the polymorphic site rs12979860. In the HIV/HCV cohort, frequent serum sampling was completed for HCV RNA and IFN levels. DNA microarray of peripheral blood mononuclear cells and individual expression of IFN-stimulated genes (ISGs) were quantified on IFN therapy. The *IL28B*-favorable (CC) genotype was associated with improved therapeutic response compared with unfavorable (CT or TT) genotypes. Patients with a favorable genotype had greater first- and second-phase viral kinetics ($P = 0.004$ and $P = 0.036$, respectively), IFN maximum antiviral efficiency ($P = 0.007$) and infected cell death loss ($P = 0.009$) compared with unfavorable genotypes. Functional annotation analysis of DNA microarray data was consistent with depressed innate immune function, particularly of natural killer cells, from patients with unfavorable genotypes ($P < 0.004$). Induction of innate immunity genes was also lower in unfavorable genotypes. ISG expression at baseline and induction with IFN was independent of *IL28B* genotype. *Conclusion:* Carriers of the *IL28B*-favorable genotype were more likely to have superior innate immune response to IFN therapy compared with unfavorable genotypes, suggesting that the unfavorable genotype has aberrant baseline induction of innate immune response pathways resulting in impaired virologic response. *IL28B* genotype is associated with more rapid viral kinetics and improved treatment response outcomes independent of ISG expression.** (HEPATOLOGY 2012;56:444-454)

Hepatitis C virus (HCV) infects more than 170 million people worldwide.[1] In resource-rich countries, HCV remains the leading cause of end-stage liver disease and hepatocellular carcinoma and is the main indication for liver transplantation.[1] In the United States and Europe, HCV affects 15%-30% of human immunodeficiency virus (HIV)-infected individuals and currently represents a major

*Abbreviations: EVR, early virological response; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN, inteferon;* IL28B, *interleukin-28B; ISG, IFN-stimulating genes; NK, natural killer; NR, nonresponder; PBMC, peripheral blood mononuclear cell; PEG-IFN, pegylated inteferon; RVR, rapid virological response; SVR, sustained virological response; VR, virologic responder.*

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cause of morbidity and mortality in the coinfected population.[2,3] Therapy for chronic hepatitis C with pegylated interferon (PEG-IFN) plus ribavirin is poorly tolerated, and a significant number of patients who receive therapy are not cured.[2,4] As a result, attempts to individualize therapy according to baseline markers and on treatment virological response are being increasingly pursued.

Three independent genome-wide association studies identified several single nucleotide polymorphisms around the interleukin-28B (*IL28B*) gene that are strongly associated with treatment outcomes in HCV-monoinfected individuals.[5-7] One of the single-nucleotide polymorphisms, rs12979860, is located on chromosome 19q13, 3 kb upstream of the *IL28B* gene, which codes for IFN-$\gamma$-3.[5] The rs12979860-favorable (CC) genotype is associated with a more than two-fold increased rate of sustained virological response (SVR) than the unfavorable (CT or TT) genotypes, a finding that is consistent across multiple ethnic groups.[5-7] This association has also been confirmed in the HIV/HCV-coinfected population.[8] Although the causal variant and biological mechanism responsible for this association has not yet been identified, recent studies have suggested that baseline hepatic IFN-stimulated gene (ISG) expression is associated with genetic variation of *IL28B*.[9,10] Meanwhile, two studies reported that ISG expression is associated with SVR independent of *IL28B* genotype.[11,12] High baseline expression of ISGs and lack of induction of ISGs have also been described as strong negative predictors of achieving SVR among HCV-monoinfected and HIV/HCV-coinfected subjects.[13,14]

In this study, we sought to further investigate the biological mechanism of the *IL28B* polymorphism rs12979860, specifically as it relates to very early viral kinetics, pharmacodynamics, and gene expression in HIV/HCV-coinfected and HCV-monoinfected patients undergoing treatment with PEG-IFN plus ribavirin.

## Patients and Methods

### *Study Subjects*

***Group A: HIV/HCV-Coinfected Cohort.*** Three prospective, single-center, open-label trials were performed at the National Institute of Allergy and Infectious Diseases, National Institutes of Health, from 2001 to 2010. Fifty HCV treatment–naïve HIV/HCV genotype 1–infected patients were treated with weight-based ribavirin daily in addition to either weekly PEG-IFN–alfa-2b at 1.5 $\mu$g/kg, weekly PEG-IFN–alfa-2a, or albinterferon–alfa-2b at 900 $\mu$g every 2 weeks. All patients received 48 weeks of therapy and follow-up for 24 weeks after completion of treatment. Clinical endpoints were defined as follows: rapid virological response (RVR), undetectable HCV RNA at week 4; partial early virological response (EVR), $\geq$2 $\log_{10}$ decline in HCV RNA at week 12; complete EVR, undetectable HCV RNA at week 12; SVR, undetectable HCV RNA 6 months after the completion of therapy; nonresponse (NR), failure to achieve HCV RNA decline $\geq$2 $\log_{10}$ at week 12 or detectable viral load at week 24 or anytime after week 24 while on therapy; relapse, evidence of a detectable viral load following the completion of therapy in a patient with an undetectable viral load at the end of treatment; virologic breakthrough, evidence of detectable viral load in a patient with a prior undetectable viral load who is still receiving therapy.

All patients gave written informed consent approved by the National Institute of Allergy and Infectious Diseases Institutional Review Board before enrollment in the studies. Intensive serum monitoring was completed at study visits day 0, 1, 3, 5, 7; week 2, 3, 4, 6, 8; and every 4 weeks until week 48 (end of treatment). Peripheral blood mononuclear cell (PBMC) collection occurred at baseline and at week 4, drawn as a trough, before the fifth IFN injection.

***Group B: HCV-Monoinfected Cohort.*** A total of 47 patients monoinfected with HCV genotype 1 were treated at the University of Essen (Essen, Germany) with weight-based ribavirin daily in addition to either weekly PEG-IFN–alfa-2b at 1.5 $\mu$g/kg (Peg-Intron, Schering-Plough) or weekly PEG-IFN–alfa-2a (Pegasys, Roche Laboratories). Clinical endpoints were the same as described for the HIV/HCV-coinfected cohort.

All patients gave written informed consent approved by the local ethics committee prior to enrollment. Serum monitoring was completed at study visits day 0 and 1 and weeks 4, 12, 24, and 48 (end of treatment). PBMC collection occurred at baseline and 12 hours after the first IFN injection.

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*Potential conflict of interest: Eva Herrmann serves as a research consultant for Gilead Sciences, Roche Pharma, and Novartis. John McHutchison and Alex Thompson are coinvestigators on the* IL28B *rs12979860 patent.*

*Additional Supporting Information may be found in the online version of this article.*

### *Laboratory Evaluations*

Safety laboratory tests and immune profiles including $CD4^+$ T cell counts were obtained at baseline and at each study visit. For both cohorts, HCV RNA concentration in plasma was measured using the VERSANT HCV RNA 3.0 Assay (Bayer Diagnostics, Puteaux, France), which has a quantitation range of 615 to $7.7 \times 10^6$ HCV RNA IU/mL.

***rs12979860 Single-Nucleotide Polymorphism Genotyping.*** Genotyping of both cohorts was performed by the Duke Center for Human Genome Variation. Genotyping was conducted in a blinded fashion on DNA specimens collected from each individual, using the 5′ nuclease assay with allele-specific TaqMan probes (ABI TaqMan allelic discrimination kit and the ABI7900HT Sequence Detection System; Applied Biosystems, Carlsbad, CA). Genotyping calls were manually inspected and verified before release.

***Measurement of Serum IFN Concentrations.*** In the HIV/HCV-coinfected cohort, the concentration of IFN was determined by application of a quantitative sandwich enzyme-linked immunosorbent assay method (Bender MedSystems Diagnostics GmbH, Vienna, Austria) as described.[15]

***DNA Microarray Analysis.*** Gene expression was assessed in both cohorts at baseline and after IFN dosing (Cohort A: prior to 5th IFN dose; Cohort B: 12 hours after 1st IFN dose). PBMCs were analyzed using Affymetrix U133A 2.0 oligonucleotide arrays according to the specified manufacturer protocols (Affymetrix, Santa Clara, CA). A significant analysis of microarray algorithm was used to determine the genes that were differentially expressed after an extensive filtering process. Genes that exhibited low variability or had expression levels for $\geq$50% of samples below the detection level were removed from the analysis. A guanosine-cytosine robust multiarray (GC-RMA) algorithm was used for preprocessing of the raw data. By eliminating those genes that had GC-RMA values with an interquartile range of $<$0.263 or a 75th percentile of $<$5; 7,527 of the initial 22,000 genes were retained for the subsequent analysis.

***Branched DNA Multiplex Assay.*** Validation of DNA microarray data was performed using a novel customized branched DNA multiplex assay capable of detecting the expression of 35 genes as described.[14,15]

### *Analyses*

***Modeling Viral Kinetics, Pharmacokinetics, and Pharmacodynamics.*** Clinical pharmacokinetic and viral kinetic data of each individual patient was fitted with a full pharmacokinetics/pharmacodynamics model.[16] Pharmacokinetics of PEG-IFN was modeled by a Bateman function using parameters $k_a$ and $k_e$ for drug absorption and elimination, respectively, as well as $FD/V_d$ for the bioavailable drug[17,18] (see Supporting Information). To analyze the observed HCV RNA decline patterns, drug effectiveness ($\epsilon$) was varied across PEG-IFN drug levels. Therefore, mean and maximum efficacy was used as summarizing measures for the efficacy of antiviral treatment. Further parameters describing the dynamics are (1) $\rho$, describing the antiviral effect of ribavirin to split the newly produced virus in infectable and uninfectable virus ($V$ and $V_n$, respectively)[19]; (2) p, describing the viral production rate in the untreated chronic patient; (3) c, describing viral clearance; and (4) $\delta$, describing infected cell loss. For data fitting, we fitted the pharmacokinetic and the logarithmized viral kinetic model function to individual patient data by a maximum likelihood approach (see Supporting Information).

***Statistical Analysis.*** All clinical outcomes were assessed in an intention-to-treat analysis. Comparisons between groups were performed using nonparametric analysis of variance. Associations between different qualitative parameters were explored using a chi-square test or Fisher's exact test, as appropriate. Univariable (chi-square test) and multivariable (logistic or linear regression) analyses were used to determine predictors of treatment outcome and viral kinetics. Gene expression data were analyzed by supervised learning (class prediction) with linear regression analysis (one-way analysis of variance) to identify gene expression profiles correlating with the prespecified outcomes (*IL28B* genotype, treatment response). For all gene expression analyses, treatment response groups were defined as nonresponder (NR, virologic breakthrough + non-responder) or virologic responder (VR, sustained virologic response + relapser). All $P$ values were two-tailed and were considered significant when less than 0.05. Statistics and graphics were performed using PARTEK Genomics Suite (St. Louis, MO) and GraphPad Prism 4 (La Jolla, CA), respectively.

## Results

### *Study Population Characteristics and* IL28B *Polymorphism*

***HIV/HCV-Coinfected Cohort.*** Of the 50 HIV/HCV-coinfected patients meeting study entry criteria, six did not have adequate stored DNA sufficient for genotyping; thus, the final analysis comprised 44 patients. The cohort was predominantly male (89%) and African-American (55%), with a median age of 48

**Table 1. Baseline Characteristics of Clinical Cohorts**

| | HIV/HCV Coinfection | | | | HCV Monoinfection | | | |
|---|---|---|---|---|---|---|---|---|
| **Baseline Characteristic** | **All (N = 44)** | **CC (n = 15)** | **CT/TT (n = 29)** | ***P*** | **All (N = 44)** | **CC (n = 11)** | **CT/TT (n = 33)** | ***P*** |
| Age, years, median (IQR) | 48 (42-52) | 48 (39-52) | 49 (43-52) | 0.321 | 43 (35-53) | 49 (36-49) | 42 (34-54) | 0.626 |
| Male sex, n (%) | 39 (89) | 14 (93) | 25 (86) | 0.647 | 23 (52) | 3 (27) | 13 (65) | 0.084 |
| Nonwhite race/ethnicity, n (%) | 30 (68) | 6 (40) | 24 (83) | 0.007 | 5 (11) | 0 | 5 (15) | 0.309 |
| Median HCV RNA ($log_{10}$ IU/mL) | 5.30 | 6.30 | 6.30 | 0.384 | 5.88 | 6.10 | 5.72 | 0.268 |
| HCV RNA >800,000 IU/mL (%) | 35 (80) | 11 (73) | 24 (83) | 0.555 | 23 (52) | 9 (81) | 14 (42) | 0.175 |
| CD4 cells/μL, median (IQR) | 550 (394-763) | 547 (396-747) | 553 (392-907) | 0.464 | – | – | – | – |
| Suppressed HIV RNA | 31 (70) | 11 (73) | 20 (69) | 0.999 | – | – | – | – |
| HAI fibrosis stage, n (%) | | | | 0.528 | | | | 0.999 |
| F0-F2 | 26 (59) | 8 (53) | 18 (62) | | 36 (81) | 9 (82) | 27 (82) | |
| F3-F4 | 18 (41) | 7 (47) | 11 (38) | | 8 (19) | 2 (18) | 6 (18) | |
| IFN formulation, n (%) | | | | 0.690 | | | | 0.719 |
| 2b 1.5 μg/kg/week | 24 (55) | 9 (60) | 15 (52) | | 16 (36) | 3 (27) | 13 (39) | |
| 2a 180 μg/week | 10 (23) | 2 (13) | 8 (28) | | 28 (64) | 8 (73) | 20 (61) | |
| alpha-2b 900 μg/2 weeks | 10 (23) | 4 (27) | 6 (20) | | – | – | – | |

Abbreviation: HAI, histological activity index; IQR, interquartile range.

years (Table 1). The cohort comprised only patients with HCV genotype 1 infection, and 80% of patients had a high baseline HCV RNA level (>800,000 IU/mL), making this a very difficult to treat group. HIV disease was well controlled, with over 70% having evidence of HIV viral suppression. The distribution of the *IL28B* genotypes was similar (CC, 35%; CT, 29%; TT, 36%). The frequency of the *IL28B* genotype differed among ethnic groups ($P = 0.005$), with the favorable genotype observed most frequently in Caucasians (64%) compared with African-Americans (17%), similar to other studies.[5]

***HCV-Monoinfected Cohort.*** Of the 47 HCV monoinfected patients meeting enrollment criteria, there was insufficient DNA for three, thus the final analysis comprised 44 patients. All patients were of German ethnicity; the majority were Caucasian (89%) and male (52%), and over half had a high HCV RNA level (>800,000 IU/mL) (Table 1). The distribution of the *IL28B* genotypes was CC 25%, CT 64%, TT 11%. None of the non-Caucasian patients had the favorable genotype.

### *Viral Clinical Endpoints*

For the HIV/HCV cohort, rates of response were similar to historical reports (RVR, 14%; EVR, 64%; SVR, 29%). Rates of clinical virological endpoints differed by *IL28B* genotypes. Patients with the favorable genotype had higher rates of complete EVR ($P = 0.009$) and SVR ($P = 0.06$); no favorable carrier was a nonresponder ($P = 0.001$). Among HCV-monoinfected patients, EVR was 78%; however, overall SVR rates were slightly lower than expected at 36%. Patients with the favorable genotype had higher SVR rates (50%) compared with unfavorable genotypes (12%) and lower rates of relapse (12% versus 29%, respectively).

### *Viral Kinetic, Pharmacokinetic, and Pharmacodynamic Parameters*

Frequent early serum monitoring was only available for 36 patients in the HIV/HCV-coinfected cohort. Although *IL28B* genetic variation had no association with pharmacokinetic parameters (Fig. 1A), there was a strong association with very early viral kinetic and pharmacodynamic parameters (Fig. 1B,C). The first-phase viral decline was greater for patients with the favorable genotype than the unfavorable genotype ($P = 0.004$), with a maximum IFN antiviral efficiency ($\epsilon_{max}$) of 95% compared with 82%, respectively ($P = 0.007$). The first-phase viral decline was not statistically associated with any virological endpoint, including RVR, EVR, SVR, and NR. The slower second-phase slope calculated from day 2/3 to day 28 was also greater for patients with the favorable genotype than the unfavorable genotype ($P = 0.036$), with a greater infected cell death/loss, $\delta$ ($P = 0.009$). Faster second-phase viral kinetics was associated with improved clinically relevant therapeutic endpoints, including RVR ($P = 0.007$), EVR ($P = 0.012$), SVR ($P = 0.03$), and NR ($P = 0.024$). In a multivariable model, only the *IL28B* polymorphism was associated with first- and second-phase slopes, whereas age, baseline HCV RNA, and baseline CD4 were not. Individual patient viral kinetics are shown in Supporting Fig. 1.

### *Impact of* IL28B *polymorphism on differential gene expression profiles in PBMCs*

To explore differential host response to IFN-based therapy for HCV across *IL28B* genotypes, we

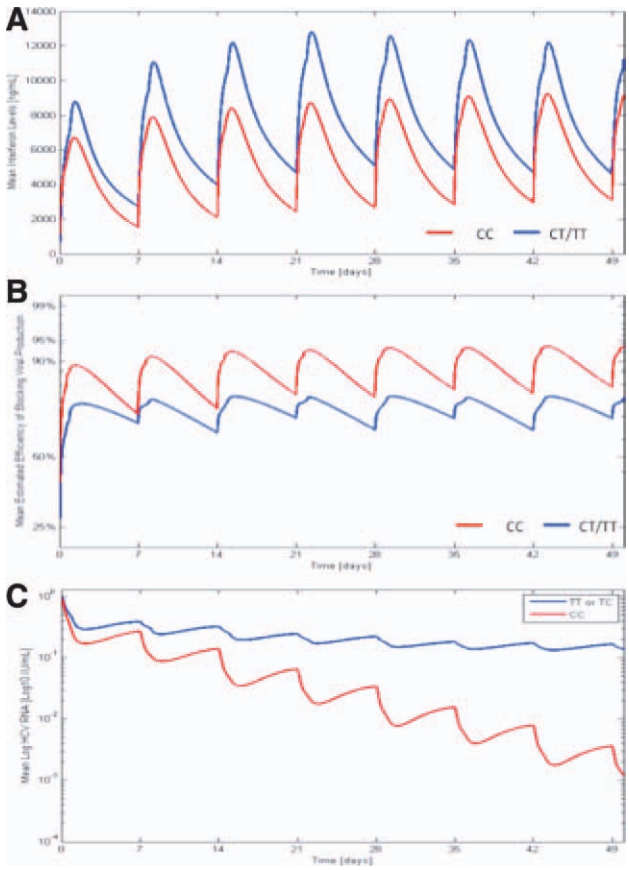


Fig. 1. Modeling. (A) Pharmacokinetics. Area under the curve of fitted pharmacokinetic function for the first week and the first 4 weeks were not significantly different by *IL28B* genotype. (B) Pharmacodynamics. Maximum efficiency (which determines first-phase viral decay) was higher for the CC-favorable genotype compared with the CT/TT-unfavorable genotype ($P = 0.007$). Mean efficiency was also higher for the CC-favorable genotype ($P = 0.061$), although this did not reach statistical significance. (C) Viral kinetics. First-phase (24 hours) and second-phase (after day 2) viral decay is greater for the CC-favorable genotype. Phases of viral decay are a function of maximum efficiency and delta (infected cell loss), respectively.

examined the gene expression profiles (baseline and after IFN dosing) in PBMCs of 20 HIV/HCV-coinfected patients (all on antiretroviral therapy, five with detectable HIV RNA [range, 100-1000 copies/mL]) and 26 HCV-monoinfected patients. The subsets of patients used in the DNA microarray analysis were chosen randomly and are representative of both cohort populations. The microarray gene expression was analyzed by dividing patients into groups (pretreatment CC, pretreatment CT/TT, pretreatment NR, pretreatment VR, posttreatment CC, posttreatment CT/TT, posttreatment NR, and posttreatment VR). Gene expression for all groups was normalized to pretreatment VR patients.

***HIV/HCV-Coinfected Cohort.*** A heat map was generated by supervised partitional clustering with reference to the baseline expression profile of 479 genes, of which 452 known unique genes were differentially regulated between favorable and unfavorable *IL28B* genotypes in coinfected subjects. We were able to identify five gene clusters that were differentially expressed among groups (Fig. 2A, Supporting Table 1). Functional annotation analysis (DAVID) led to further identification of 16 genes (Table 2) that were expressed at higher levels in unfavorable versus favorable genotypes ($P = 0.004$) and biologically implicated in innate immunity functions. These genes were overrepresented in several pathways of innate immunity and natural killer (NK) cell activity: killer inhibitory receptors, which are inhibitory for NK cells, natural cytotoxic receptors, and major histocompatibility complex class 1 complex. In contrast to the lower level of expression of this cluster of genes in the favorable genotype, their induction following exposure to exogenous IFN was increased relative to unfavorable genotypes ($P = 0.004$).

***HCV-Monoinfected Cohort.*** A heat map was generated by supervised partitional clustering with reference to the baseline expression profile of 655 genes, of which 573 known unique genes were differentially regulated between the favorable and unfavorable *IL28B* genotypes in HCV-monoinfected patients. We were able to identify seven clusters that consisted of genes that were differentially expressed among the groups (Fig. 2B, Supporting Table 2). Similar to the results observed with HIV/HCV-coinfected subjects, HCV-monoinfected subjects had an overrepresentation of innate immune response genes differentially expressed between the different genotypes (Table 3). Genes associated with cytotoxic T and NK cell inhibition, such as killer inhibitory receptors, natural cytotoxic receptors, and granzyme were expressed at higher levels in unfavorable genotype patients. In addition, there were three classes of genes that were differentially regulated in HCV-monoinfected subjects not seen in the HIV/HCV-coinfected cohort: (1) several genes that belonged to the class of apoptosis or cell death, specifically the caspase pathway, were up-regulated in patients with the favorable genotype; (2) there was overexpression of the IL27 receptor in patients with an unfavorable genotype; and (3) patients with an unfavorable genotype had higher expression of several representative genes belonging to the ras family of oncogenes (RAB35, RAP2A, RASA4, RAB4B, NFKIRAS2). Of the 452 and 573 known unique genes that were differentially expressed in PBMCs of HIV/HCV-coinfected and HCV-monoinfected patients, respectively, only a minor fraction of genes ($n = 26$) were shared between groups. These genes

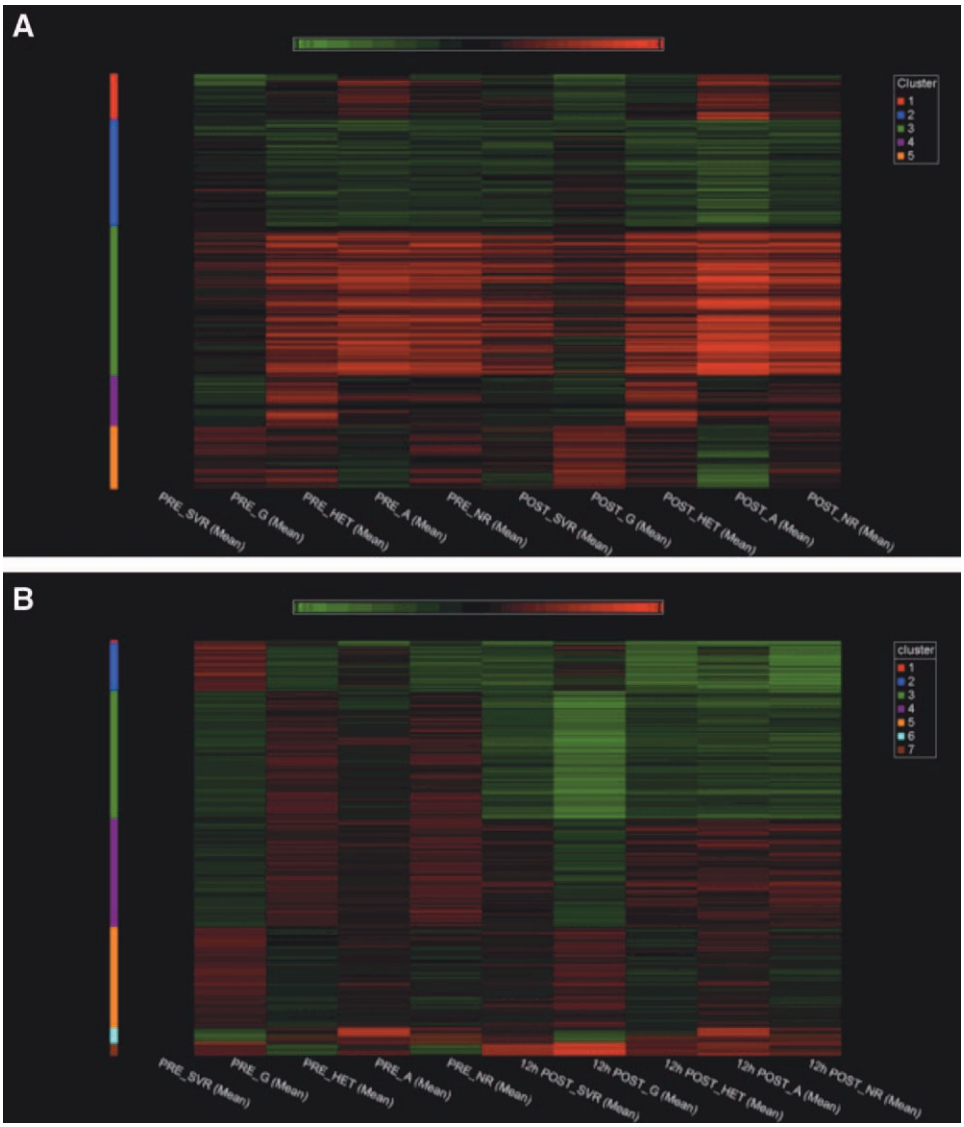


Fig. 2. DNA microarray gene expression profiles. (A) HCV/HIV coinfection cohort (n = 20). PRE = baseline before IFN therapy; POST = trough after the fourth dose of IFN therapy; G = favorable genotype (CC); HET = unfavorable genotype (CT); A = unfavorable genotype (TT); NR = nonresponder to IFN therapy; SVR = responder to IFN therapy. Cluster 1 genes were up-regulated in both NR and unfavorable genotypes at PRE and POST time points. These genes belong to regulation of both adaptive and innate immune responses (major histocompatibility complex class 1 receptor activity, $P = 4.7 \times 10^{-8}$; genes involved in antigen processing and presentation, $P = 1.2 \times 10^{-7}$; NK cell–mediated cytotoxicity, $P = 1.9 \times 10^{-7}$). Cluster 2 genes were up-regulated in the favorable but not unfavorable genotypes at PRE and POST time points, with the major functional category being genes involved in a host-virus interaction ($P = 3.4 \times 10^{-4}$) and epigenetic modification of DNA ($3.5 \times 10^{-3}$). Cluster 3 genes were up-regulated in all groups compared with baseline expression in the SVR group and represent posttranslational modification and protein metabolism genes ($P = 7.1 \times 10^{-9}$), suggesting an increased metabolic state in these patients compared with SVR. Cluster 4 included genes up-regulated in NR or unfavorable genotypes at both PRE and POST time points. This cluster revealed up-regulation of genes directly involved in tissue injury and inflammatory response ($P = 3.1 \times 10^{-4}$), which may reflect sustained tissue damage without HCV clearance. Cluster 5 genes were expressed at higher levels among those who were NR or had the favorable genotype. This cluster included protein transport, endosomal, and lysosomal functions ($P = 6.1 \times 10^{-4}$) and is of unclear significance. (B) HCV monoinfection cohort (n = 26). PRE = baseline before IFN therapy; POST = 12 hours after first dose of IFN therapy; G = favorable genotype (CC); HET = unfavorable genotype (CT); A = unfavorable genotype (TT); NR = nonresponder to IFN therapy; SVR = responder to IFN therapy. Cluster 2 included the genes that were up-regulated in both PRE and POST treatment samples of the favorable genotype. The functional category of genes in this cluster included many coding for the ras pathway genes ($P = 1 \times 10^{-8}$). Cluster 3 comprised genes up-regulated only in unfavorable genotypes or NRs at PRE but not POST time points and included phosphoproteins involved in cellular signaling and activation ($P = 1.5 \times 10^{-8}$). Cluster 4 genes were up-regulated only in unfavorable genotypes or NRs at PRE and POST time points. These genes clearly induced cellular signaling pathways and RNA editing ($P = 3.1 \times 10^{-7}$ and p1 $\times 10^{-3}$ respectively). Cluster 5 genes were up-regulated at PRE and POST time points for all *IL28B* genotypes but were different from NRs. These genes are involved in cellular signal transduction and histone modulation ($P = 4.2 \times 10^{-5}$) with unclear significance. Cluster 6 genes were up-regulated in subjects who were NR or unfavorable genotypes at PRE and POST time points. Genes encoding structural and microtubule formation were clearly overrepresented in this cluster ($P = 6.7 \times 10^{-5}$). Cluster 7 included genes that were expressed at higher levels in favorable genotype rather than SVR or unfavorable genotype. Interestingly, these genes included those involved in protection from cell death and apoptosis and negative regulation of apoptotic pathway ($P = 6.9 \times 10^{-4}$).

**Table 2. Differentially Regulated Innate Immunity Genes In HIV/HCV Coinfection By *IL28B* Haplotype**

| Affymetrix ID | Gene Name | Symbol | Regulation | Function |
|---|---|---|---|---|
| 207313_at | killer cell immunoglobulin-like receptor, long cytoplasmic tail, 1 | KIR3DL2 | Up in CT/TT | Inhibitory for NK cells |
| 211532_x_at | killer cell immunoglobulin-like receptor, short cytoplasmic tail, 1 | KIR2DS1 | Up in CT/TT | Inhibitory for NK cells |
| 210606_x_at | killer cell lectin-like receptor subfamily D, member 1 | KLRD1 | Up in CT/TT | Inhibitory for NK cells |
| 211583_x_at | natural cytotoxicity triggering receptor 3 | NCR3 | Up in CT/TT | Activating for NK cells |
| 208203_x_at | killer cell immunoglobulin-like receptor, short cytoplasmic tail, 5 | KIR2DS5 | Up in CT/TT | Inhibitory for NK cells |
| 206785_s_at | killer cell lectin-like receptor subfamily C, member 1 | KLRC1 | Up in CT/TT | Inhibitory for NK cells |
| 211528_x_at | major histocompatibility complex, class I, G | HLA-G | Up in CT/TT | Inhibitory for NK cells |
| 214459_x_at | major histocompatibility complex, class I, C | HLA-C | Up in CT/TT | Inhibitory for NK cells |
| 217436_x_at | major histocompatibility complex, class I, A | HLA-A | Up in CT/TT | Inhibitory for NK cells |
| 205488_at | granzyme A | GZMA | Up in CT/TT | Cell cytotoxicity |
| 37145_at | granulysin | GNLY | Up in CT/TT | Cell cytotxicity |
| 213033_s_at | nuclear factor I/B | NFIB | Up in CT/TT | Cell signaling |
| 205718_at | integrin, beta 7 | ITGB7 | Up in CT/TT | Cell migration |
| 203828_s_at | interleukin 32 | IL32 | Up in CT/TT | Proinflammatory cytokine |
| 209924_at | chemokine (C-C motif) ligand 18 | CCL18 | Up in CT/TT | Lymphocyte chemoattractant |
| 214049_x_at | CD7 molecule | CD7 | Up in CT/TT | T cell interactions |

held diverse functions and overall did not correlate to a well-defined cellular function.

### *Impact of* IL28B *Polymorphism on Differential ISG Expression Profiles*

Using a literature-mining algorithm that demonstrated up-regulation of IFN-stimulated genes (ISGs) by IFN *in vitro*, a select panel of ISGs (n = 20) was assessed in those HIV/HCV-coinfected patients receiving PEG-IFN alpha-2b (n = 21) at baseline and after IFN dosing. This group was selected due to differences in treatment regimens across groups and concern that this may impact ISG expression, thus the largest trial was chosen for the ISG expression analysis. Differential ISG expression was assessed by *IL28B* genotype and treatment response (NR versus VR) using a stratified algorithm.

Total baseline and delta mean ISG expression in PBMCs did not differ by *IL28B* genotype (Supporting Fig. 2). Furthermore, there were no differences in specific ISG expression between *IL28B* genotypes either at baseline or for delta gene expression (Supporting Fig. 2). Due to enrichment of VR in those patients with the *IL28B*-favorable genotype and NR in those patients with the unfavorable genotype, the analysis was also completed in subgroups stratified by either

**Table 3. Differentially Regulated Innate Immunity Genes in HCV Monoinfection by *IL28B* Haplotype**

| Affymetrix ID | Gene Name | Symbol | Regulation | Function |
|---|---|---|---|---|
| 213373_s_at | Caspase 8, apoptosis-related peptidase | CASP8 | Up in CC | Enhances apoptosis |
| 214486_x_at | CASP8- and FADD-like apoptosis regulator | CFLAR | Up in CC | Enhances apoptosis |
| 211010_s_at | Natural cytotoxicity triggering receptor 3 | NCR3 | Up in CT/TT | Inhibits NK cell cytotoxicity |
| 208179_x_at | Killer cell immunoglobulin-like receptor | KIR2DL3 | Up in CT/TT | Inhibits NK cell cytotoxicity |
| 207535_s_at | Nuclear factor of kappa | NFKB2 | Up in CT/TT | Represents cellular activation |
| 202426_s_at | Retinoid X receptor, alpha | RXRA | Up in CT/TT | Represents cellular activation |
| 206519_x_at | Sialic acid binding immunoglobulin-like lectin 6 | SIGLEC6 | Up in CT/TT | Represents cellular activation |
| 206148_at | Interleukin-3 receptor, alpha (low-affinity) | IL3RA | Up in CT/TT | Represents cellular activation |
| 215099_s_at | Retinoid X receptor, beta | RXRB | Up in CT/TT | Represents cellular activation |
| 205611_at | Tumor necrosis factor (ligand) superfamily, member 12 | TNFSF12 | Up in CT/TT | Proinflammatory response |
| 205926_at | Interleukin 27 receptor, alpha | IL27RA | Up in CT/TT | Proinflammatory response |
| 205114_s_at | Chemokine (C-C motif) ligand 3 | CCL1 | Up in CT/TT | Proinflammatory response |
| 200984_s_at | CD59, complement regulatory protein | CD59 | Up in CT/TT | Proinflammatory response |
| 207460_at | Granzyme M (lymphocyte met-ase 1) | GZMM | Up in CT/TT | Proinflammatory response |
| 207094_at | Interleukin-8 receptor, alpha | IL8RA | Up in CT/TT | Proinflammatory response |
| 205461_at | RAB35, member RAS oncogene family | RAB35 | Up in CT/TT | Inhibits IFN-alpha response |
| 202252_at | RAB13, member RAS oncogene family | RAB13 | Up in CT/TT | Inhibits IFN-alpha response |
| 204010_s_at | v-Ki-ras2 Kirsten rat sarcoma oncogene | KRAS | Up in CT/TT | Inhibits IFN-alpha response |
| 219167_at | RAS-like, family 12 | RASL12 | Up in CT/TT | Inhibits IFN-alpha response |
| 219807_x_at | RAB4B, member RAS oncogene family | RAB4B | Up in CT/TT | Inhibits IFN-alpha response |
| 208534_s_at | RAS p21 protein activator 4 | RASA4 | Up in CT/TT | Inhibits IFN-alpha response |
| 214487_s_at | RAP2A, member of RAS oncogene family | RAP2A | Up in CT/TT | Inhibits IFN-alpha response |
| 222105_s_at | NFKB inhibitor interacting Ras-like 2 | NKIRAS2 | Up in CT/TT | Inhibits IFN-alpha response |

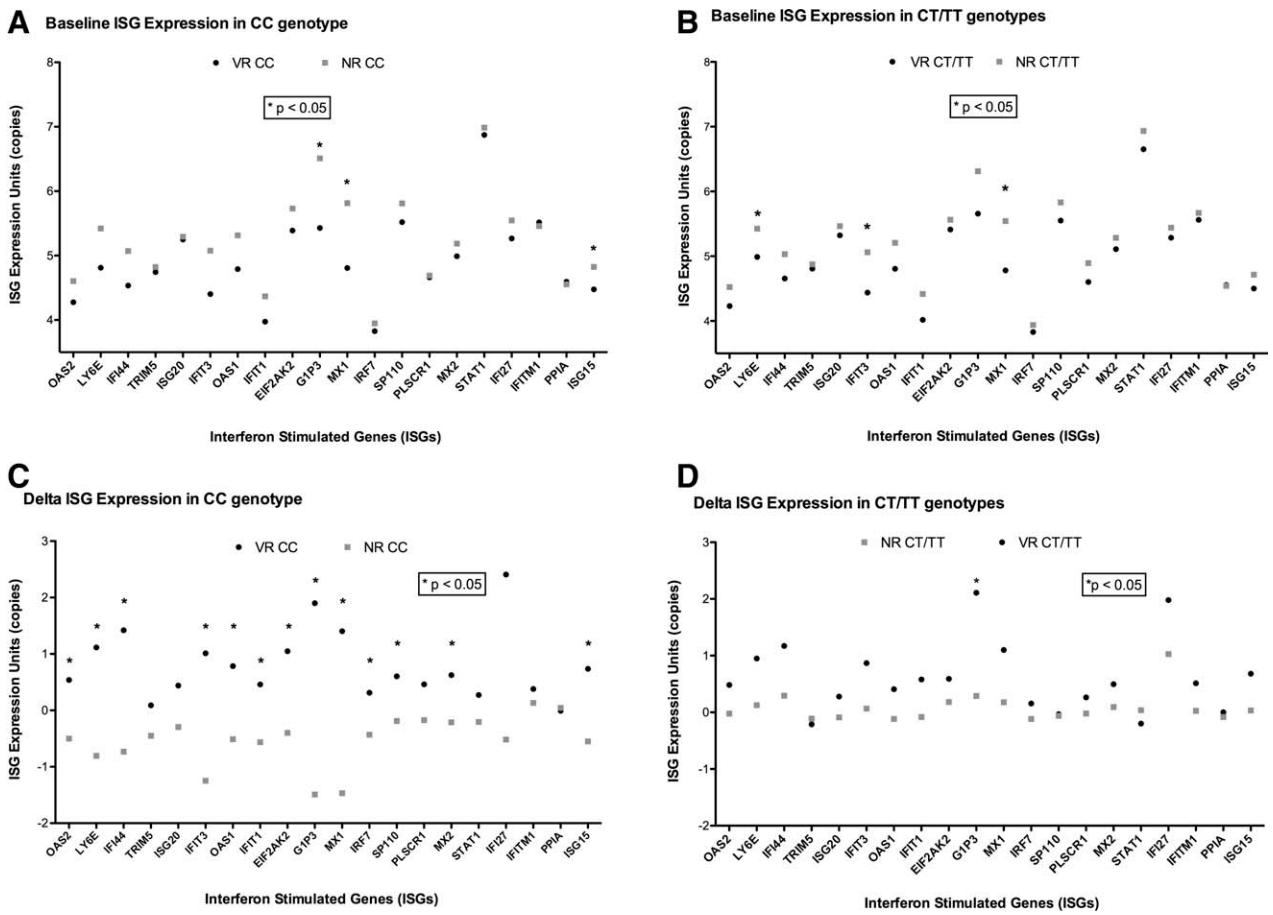


Fig. 3. Individual ISG expression for treatment response stratified by *IL28B* genotype. (A) Mean baseline ISG expression in the favorable (CC) genotype. (B) Mean baseline ISG expression in the unfavorable (CT/TT) genotype. (C) Mean delta ISG expression in the favorable (CC) genotype. (D) Mean delta ISG expression in the unfavorable (CT/TT) genotype.

*IL28B* genotype or treatment response. When treatment response (VR or NR) was stratified by *IL28B* genotype (CC or CT/TT), there was no difference in total mean baseline ISG expression (Fig. 3A). At baseline, the NR-favorable (CC) subgroup did have higher G1P3 (a mitochrondrial protein with reported antiapoptotic activity) and MX1 (antiviral activity reported to increase in VR after interferon therapy) gene expression levels ($P < 0.05$) than the VR-favorable subgroup (Fig. 4A). In addition, the NR-unfavorable (CT/TT) subgroup had higher lymphocyte antigen 6 complex (LY6E), transmembrane protein with antiviral activity (IFIT3), and MX1 gene expression levels ($P < 0.05$) than the VR-unfavorable subgroup (Fig. 4B). Conversely, the total mean delta ISG expression was significantly different across treatment response groups, regardless of *IL28B* genotype (Fig. 3B). This change in ISG expression was most evident in those patients with the *IL28B*-favorable genotype (Figs. 4C,D). Both VR-favorable ($P < 0.0001$) and VR-unfavorable ($P = 0.001$) subgroups had higher total mean delta ISG expression after IFN dosing than the NR-favorable and NR-unfavorable subgroups, respectively (Fig. 3B). The increase in ISG gene expression for VR stratified by *IL28B* genotype was not different ($P > 0.05$) (Fig. 3B). When *IL28B* genotype (CC or CT/TT) was stratified by treatment response (VR or NR), there was no difference in mean baseline ISG expression (Supporting Fig. 3).

### *African American Patients Have Higher ISG Expression at Baseline and Lower Levels of Induction of ISGs with IFN Therapy Independent of* IL28B *Genotype*

We examined the effect of *IL28B* genotype on ISG expression in both African American and Caucasian subjects who were coinfected with HIV and HCV. African Americans had a higher level of ISG expression at baseline and a lower level of ISG induction after IFN dosing compared with Caucasians of any *IL28B* genotype (Fig. 5). African Americans with the more favorable genotype failed to induce ISGs, with levels remaining lower than Caucasians with the less favorable CT/TT genotypes.

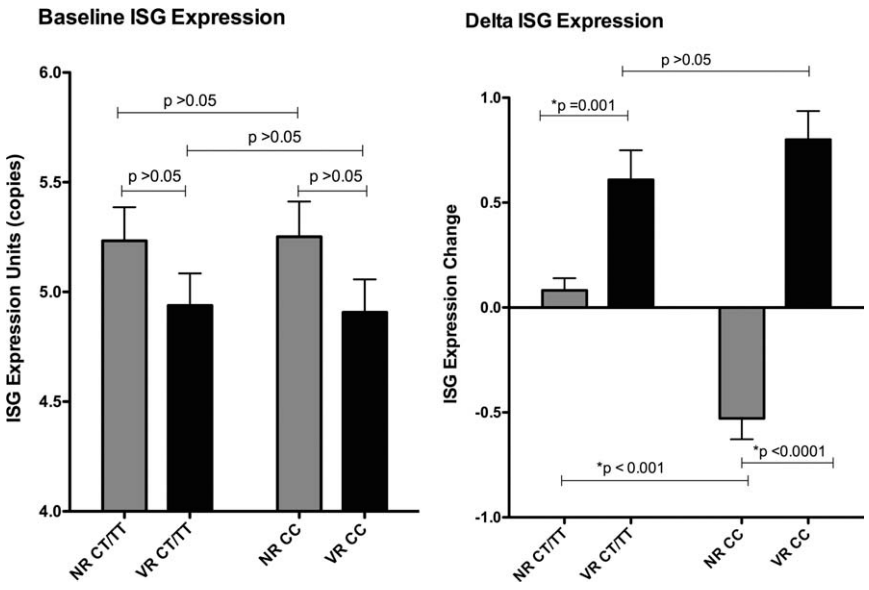


Fig. 4. Total ISG expression for treatment response stratified by *IL28B* genotype. (A) Mean baseline ISG expression. (B) Mean delta ISG expression.

## Discussion

We explored the influence of the *IL28B* polymorphism on therapeutic responses to HCV treatment and the patterns of gene expression in PBMCs at baseline and after early exogenous IFN exposure. We found that the *IL28B*-favorable CC genotype is strongly associated with improved clinical outcomes, viral kinetics, and pharmacodynamic parameters. Global gene expression analyses identified dysregulation in several pathways of innate immunity and NK cell activity in patients with the unfavorable *IL28B* genotype, resulting in a muted response to IFN therapy. Conversely, a focused analysis of gene expression suggested that *IL28B* genotype and ISG expression are independent predictors of IFN responsiveness. To the best of our knowledge, these are the first data investigating the relationship between *IL28B* genotype and patterns of gene expression both at baseline and while on IFN therapy.

The detailed viral kinetic analysis showed that the favorable genotype is associated with more rapid first- and second-phase viral decline. The more rapid first-phase viral decline in patients with the favorable allele suggests that IFN has a greater effect on blocking viral production and release of virions by infected cells. The more rapid second-phase viral kinetics seen in patients with the favorable allele also raises the prospect of improved cell-mediated immunity and a more robust immune response resulting in more rapid loss of infected hepatocytes. These findings are similar to those reported by other groups.[20-22] *IL28B* genotype was the only independent factor associated with early

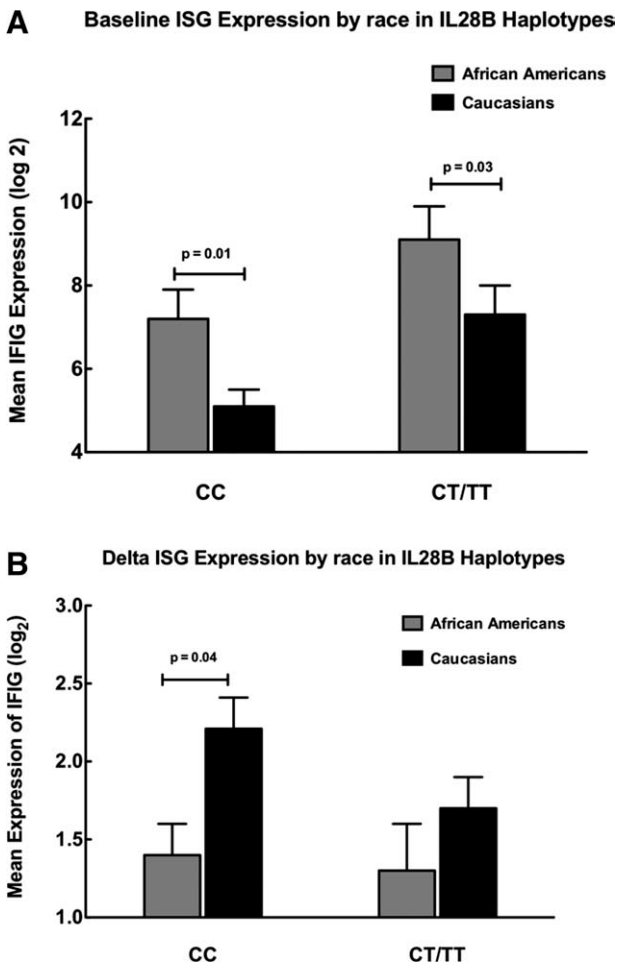


Fig. 5. Total ISG expression by race and *IL28B* genotype. (A) Baseline ISG expression by race and *IL28B* genotype. (B) Delta ISG expression by race and *IL28B* genotype.

viral kinetic parameters, further advocating that the mechanistic role of this genetic variation lies not only in IFN-boosted innate immune response but adaptive response to HCV infection as well.

The association of *IL28B* genetic variability and innate immune response is further supported by the mechanistic gene expression data reported here. Patients with the *IL28B*-favorable genotype had lower baseline expression of several pathways of innate immunity and NK cell activity but more significant induction after IFN exposure compared with the unfavorable genotypes. These data are in agreement with a recent report identifying higher pretreatment levels of NK inhibitory receptors in patients with the unfavorable genotype.[23] Taken together, these findings support the hypothesis that the innate immune response in patients with the unfavorable genotype is aberrantly regulated resulting in an impaired treatment response. This dysregulation of innate immune function in the unfavorable genotypes was noted in the HIV/HCV-coinfected and HCV-monoinfected cohorts. In the HCV cohort, further immune dysregulation in the unfavorable genotype was noted in genes representative of the caspase and ras family of oncogenes canonical pathways.

Extending this investigation to select ISGs known to be up-regulated in response to IFN, we found that the *IL28B* genotype did not appear to predict ISG expression, either at baseline or on induction with IFN exposure. In a stratified analysis according to treatment response and *IL28B* genotype, ISG expression varied between response groups irrespective of their *IL28B* genotype. The greatest difference in variation was noted in the favorable genotype on induction by IFN, with VR having greater induction than the NR counterparts. These findings are similar to those reported by Dill et al.[11] and Asahina et al.[12]; both studies suggest that *IL28B* genotype does not determine ISG expression. Rather, these data suggest that ISG expression and *IL28B* genotype are both independently associated with treatment response. Unlike the others, we were unable to show that treatment response or *IL28B* genotype was associated with total mean baseline ISG expression, but this is more likely a result of our small sample size and possibly a lower sensitivity to detect variation in total ISG expression due to the use of PBMCs instead of liver tissue. Similar to prior reports, we did find several ISGs that were up-regulated at baseline in the NR group, specifically MX1, G1P3, LY6E, IFIT3.

The relationship of ISG expression and *IL28B* genotype was further explored by examining differential ISG expression stratified by race. Patients of African descent have been reported to have lower IFN response rates when compared with their European descent counterparts. The *IL28B* polymorphism has been reported to explain 50% of the variation seen in IFN response rates due to African American race.[5] Here we show that African Americans have higher baseline ISG expression and no significant ISG induction following IFN therapy independent of their *IL28B* genotype. In fact, African Americans with favorable genotype had similar ISG expression to Caucasians with the unfavorable genotype. These data further support the hypothesis that there are other factors independent of *IL28B* genetic variations that influence ISG expression.

The paradox of poor virologic response and higher baseline ISG expression has been reported but is poorly understood. Genomic studies have suggested that innate immune activation and ras pathway gene activation are the two canonical pathways most associated with lack of response to IFN in HIV/HCV-coinfected and HCV-monoinfected patients, respectively.[24,25] Given that *IL28B* genotypes have been shown to be responsible for protection against chronic HCV infection,[26] it is plausible that this genetic haplotype may determine the susceptibility of infectious organisms to innate immune defense mechanisms, particularly those mediated by NK cells. Furthermore, the role of the ras pathway in HCV has been reported to enable the establishment of persistent infection.[27] We report that the unfavorable genotype was associated with overexpression of several ras pathway genes, suggesting another potential mechanism of nonresponse among HCV-infected unfavorable genotype individuals.

There are several limitations to this study, including the small sample size and the overall heterogeneity of the cohorts. We observed minimal overlap of gene expression transcripts between the HIV/HCV-coinfected and HCV-monoinfected cohorts, a finding for which there are at least two explanations. First, PBMCs were collected at different time points for both cohorts in relation to the IFN dosing and time on IFN treatment, thus the HIV/HCV-coinfected cohort, which had received multiple doses of IFN, may have had overall down-regulated IFN-associated gene expression, as has been suggested previously.[28] Second, the use of PBMCs instead of liver tissue could lead to a greater difference in gene expression profiles due to HIV coinfection. Other limitations to this study include the lack of available liver tissue for assessment of organ-specific gene expression. Although there is overlap of gene expression between PBMCs and liver tissue, PBMCs have less ISG

induction, thus increasing the risk of type 2 error when comparing between *IL28B* genotypes. Similarly, the use of PBMCs confers risk of type 2 error in assessing broad gene expression profiles, due to the much greater gene expression detected in PBMC, thus there is a lack of specificity.

In conclusion, our data provide further insight into the host gene regulation of IFN responsiveness. The *IL28B* polymorphism is associated with improved very early viral kinetic parameters, which translates to improved clinical outcomes. Significant variation was noted across *IL28B* genotypes in innate immunologic response pathways such as killer inhibitory receptor and NK cell activation and adaptive immunity by way of increased antigen presentation and T cell activation, as well as cell signaling and cell death/apoptosis pathways. Meanwhile, the effect of *IL28B* genotype and ISG expression on treatment response appear to be independent. Although exploratory in nature, this study suggests that there are cellular and immunologic differences between *IL28B* genotypes that are independent of ISG expression; further investigation into those pathways may shed more light on the primary mechanism of this genetic variation.

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